

# Identification of D-Sorbitol 3-Phosphate in the Normal and Diabetic Mammalian Lens\*

The  $^{31}\text{P}$  NMR spectra of mammalian lenses show, in addition to previously identified compounds such as ATP,  $\text{P}_i$ , glycerophosphorylcholine, and  $\alpha$ -glycerophosphate, an unusual phosphorylated metabolite resonating at 6.5 ppm. The concentration of this compound increases manyfold in the lenses of diabetic rats concomitant with activation of the aldose reductase pathway.

We have purified this material and have identified it as D-sorbitol 3-phosphate. It appears likely that this unusual metabolite of sorbitol may reflect or be part of the metabolic aberration of diabetes in the lens.

A recent  $^{31}\text{P}$  NMR analysis of crystalline lens (1) has shown dramatic differences in the spectra of normal *versus* diabetic rats. The most striking difference was the accumulation in the diabetic lens of  $\alpha$ -glycerophosphate and two unidentified compounds with phosphorus resonances at 5.8 and 6.5 ppm. In related experiments, isolated rabbit lenses incubated in high glucose media also showed significant increases in the concentrations of  $\alpha$ -glycerophosphate and the unknown with a  $^{31}\text{P}$  resonance at 6.5 ppm (2, 3). These elevations in the concentrations of  $\alpha$ -glycerophosphate and the unidentified compounds were suppressed by treatment with the aldose reductase inhibitor sorbinil (4), suggesting that they arise via the aldose reductase-associated polyol pathway (5). This pathway produces fructose 1-phosphate from glucose via an NADPH-linked reduction of glucose to sorbitol (6, 7) and an NAD-linked oxidation of sorbitol to fructose (8) followed by phosphorylation of fructose 1-phosphate by ketohexokinase (9). Its activation has been associated with diabetic complications in diabetes mellitus (10).

The unidentified compounds, while clearly phosphate monoesters, resonate at least 1.0 ppm further downfield from other phosphate monoesters. Such  $^{31}\text{P}$  chemical shifts have not been reported previously for any known compounds of this class. Since one of these compounds (6.5 ppm) is found in normal mammalian lenses (11), we have used calf lenses as a convenient source for its purification. The purified material was characterized by a variety of techniques, mostly

NMR, and was identified as D-sorbitol 3-phosphate. This identification was confirmed by two independent chemical syntheses: (a) reduction of glucose 3-phosphate, and (b) phosphorylation of sorbitol.

## EXPERIMENTAL PROCEDURES

**Materials**—Lenses were excised from calf eyes obtained from a commercial slaughterhouse. G-10 and G-25 Sephadex gel filtration media, sorbitol dehydrogenase, NAD, and all common chemicals were purchased from Sigma. DEAE-Trisacryl<sup>TM</sup> was obtained from LKB-Pharmacia Biotechnology Inc.

**Purification of the Unknown**—The excised lenses were weighed and homogenized in 4 volumes of cold 4.2% (w/w) perchloric acid in 40% EtOH for 1 min using a Tissumiser<sup>TM</sup>. An additional 4 volumes of 2.8% perchlorate was added to the homogenized suspension followed by centrifugation at  $28,000 \times g$  for 10 min. The supernatant was neutralized with 3.5 M KOH and set aside for 15 min on ice. It was centrifuged again and then was concentrated to 20 ml on a rotary evaporator at 30 °C. The concentrated solution was left overnight at 4 °C to precipitate additional  $\text{KClO}_4$ .

The crude extract was passed through a  $5 \times 25$ -cm Sephadex G-25 column to remove high molecular weight components. Fractions eluting after the void volume were pooled, concentrated to 5 ml, and then passed through a  $5 \times 45$ -cm Sephadex G-10 column to remove excess inorganic ions. Tubes containing the target compound (as assayed by  $^{31}\text{P}$  NMR) were pooled, treated with washed activated charcoal (1 mg/g of lenses), and filtered. The filtrate was then adjusted to pH 5.8 and loaded onto a  $1.5 \times 16$ -cm column of DEAE-Trisacryl (pH 5.8). The DEAE column was developed with a linear gradient of 0–250 mM ammonium formate (pH 5.8), and fractions were assayed by  $^{31}\text{P}$  NMR.

Fractions containing the unknown were pooled and evaporated several times to remove excess ammonium formate. The final yield of material was 1.5 mg of unknown from 75 g of calf lenses.

**NMR Spectroscopy**—All NMR spectra were acquired on a Bruker AM-400 spectrometer using standard equipment and software.

$^{31}\text{P}$  NMR spectra were obtained at 161.98 MHz using a 10-mm  $^{31}\text{P}$  probe and were referenced to an external standard of glycerophosphorylcholine set at 0.49 ppm. Two-dimensional heteronuclear correlated  $^{31}\text{P}$ - $^1\text{H}$  spectra were obtained using a standard sequence as described by Bax and Morris (12).  $^{13}\text{C}$  Spectra were collected at 100.65 MHz with chemical shifts referenced to tetramethylsilane at 0 ppm. The  $^{13}\text{C}$  assignments of sorbitol are due to Kieboom *et al.* (13) and Angyal and Le Fur (14). Homonuclear  $^1\text{H}$  COSY spectra were obtained using a sequence due to Aue *et al.* (15) and Nagayama *et al.* (16). Further details on acquisition parameters are given in the figure legends.

**Phosphatase Treatment**—Approximately 1 mg of the parent compound was derived from the unknown by treatment with 5 units of alkaline phosphatase at pH 9.0. The progress of the dephosphorylation reaction was assayed by  $^{31}\text{P}$  NMR.

**Gas Chromatography**—A dried sample (250  $\mu\text{g}$ ) of the dephosphorylated material was added to 100  $\mu\text{l}$  of pyridine and 100  $\mu\text{l}$  of acetic anhydride. The solution was heated at 70 °C (30 min). The solvent was evaporated under a stream of nitrogen, and the sample was taken up in 100  $\mu\text{l}$  of chloroform.

A 4- $\mu\text{l}$  aliquot of the chloroform solution was injected into a SP2330

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gas chromatography column (0.25 × 15 m) held at 220 °C for 4 min and then programmed to 250 °C at 2 °C/min. The retention time of the unknown (17.51 min) matched the retention time of the known sorbitol acetate (17.58 min).

**Gas Chromatography/Mass Spectroscopy**—The remaining chloroform solution was concentrated to 50  $\mu$ l, and 3  $\mu$ l of this solution was injected into the injection port of a Hewlett Packard HP 5995 gas chromatography mass spectrometer. The column was equipped with a 0.25 × 15-mm Ultra silicone column (Supelco, Inc.). The temperature was held at 100 °C for 4 min and then programmed to 125 °C at 6 °C/min. The 70-eV mass spectrum of the single compound with a retention time of 18.02 min showed the fragmentation ions characteristic of sorbitol acetate, i.e.  $m/e$  289, 145, 139, 128, 115, and 103.

**Determination of the Absolute Configuration**—Having established that the parent compound of the purified metabolite is sorbitol, its absolute configuration was determined using an enzymatic assay for sorbitol (17). The assay mixture consisted of 80 mM pyrophosphate buffer, pH 9.5, 3 mM NAD<sup>+</sup>, and 0.2 mM sorbitol. The reaction was initiated by the addition of 10 units of sorbitol dehydrogenase and followed by observing the increase in absorption at 340 nm due to the production of NADH. This assay discriminated between the D and L forms of sorbitol since L-sorbitol (prepared from L-glucose by reduction with NaBH<sub>4</sub>) is not a substrate. In contrast, sorbitol prepared from the purified lens material was as good a substrate as D-sorbitol.

**Synthesis of Sorbitol 3-Phosphate**—To a solution of glucose 3-phosphate (18) (0.10 g, 0.38 mmol) in water (100 ml) was added solid NaBH<sub>4</sub> (0.1-g portions) until <sup>31</sup>P NMR showed the reaction to be complete. The solution was concentrated to 20 ml and desalted on a column of G-10 Sephadex. The desalted eluent was chromatographed on a column of DEAE-cellulose (formate) (2.5 × 25 cm) employing a gradient of 0–0.2 M ammonium formate (pH 5.8, 2 liters). All fractions containing sorbitol 3-phosphate (as determined by <sup>31</sup>P NMR) were pooled and lyophilized. Several lyophilizations removed excess ammonium formate and gave the ammonium salt of sorbitol 3-phosphate as a free flowing white powder (91 mg, 81% yield). This material had identical <sup>1</sup>H, <sup>13</sup>C, and <sup>31</sup>P NMR spectra as the material isolated from the lens.

**Synthesis of Sorbitol Phosphates**—The primary hydroxyl groups of sorbitol were protected as triphenylmethyl ethers by dissolving sorbitol (0.18 g, 1 mmol) and triphenylmethyl chloride (0.61 g, 2.2 mmol) in pyridine (10 ml) and heating at 75 °C for 2 h. The solution was cooled, and pyridinium  $\beta$ -cyanoethylphosphate (1 mmol) was added followed by dicyclohexylcarbodiimide (1 g, 5 mmol) (19). The mixture was maintained at 20 °C for 48 h. Water (10 ml) was added, and after 1 h, the mixture was filtered and evaporated. The residue was treated with 80% acetic acid (25 ml) and heated at 60 °C for 5 h to remove the triphenylmethyl-protecting groups. After cooling, the mixture was filtered and evaporated. The residue was dissolved in 50% ammonium hydroxide (25 ml) and kept at 20 °C for 16 h to remove the cyanoethyl-blocking group on phosphate. The ammonia mixture was filtered and evaporated. The crude mixture of sorbitol phosphates thus obtained consisted of four positional isomers having <sup>31</sup>P resonances of 6.5, 5.1, 4.7, and 4.3 ppm at (pH 7.5). The mixture was dissolved in water and chromatographed on a DEAE-Trisacryl (formate) column (2.5 × 20 cm) with a linear gradient of ammonium formate (0–0.2 M, pH 5.8). Fractions corresponding to each isomer (as determined by <sup>31</sup>P NMR) were combined and lyophilized. The material with the <sup>31</sup>P resonance of 6.5 ppm was identical in all respects to the sorbitol 3-phosphate prepared from glucose 3-phosphate.

## RESULTS AND DISCUSSION

The proton-decoupled <sup>31</sup>P NMR spectrum of the purified compound consists of a single resonance at 6.5 ppm (pH 7.5) (Fig. 1A). In a coupled spectrum, the compound gives a doublet with a <sup>31</sup>P-<sup>1</sup>H-coupling constant of 10.5 Hz (Fig. 1B). The chemical shift position of this peak titrates with a  $pK_a$  of 5.4 (Fig. 1C), suggesting that it is a phosphate monoester. It is an unusual phosphate monoester, however, since its chemical shift of 6.5 ppm at neutral pH is at least 1.5 ppm further downfield than other known phosphate monoesters. In addition, its  $pK_a$  of 5.4 is lower than usual (normal  $pK_a$  range is 5.9–6.2 (20)).

The <sup>13</sup>C NMR spectrum of the unknown (Fig. 2A) consists of six peaks of equal intensity ranging from 63.5 to 74.5 ppm

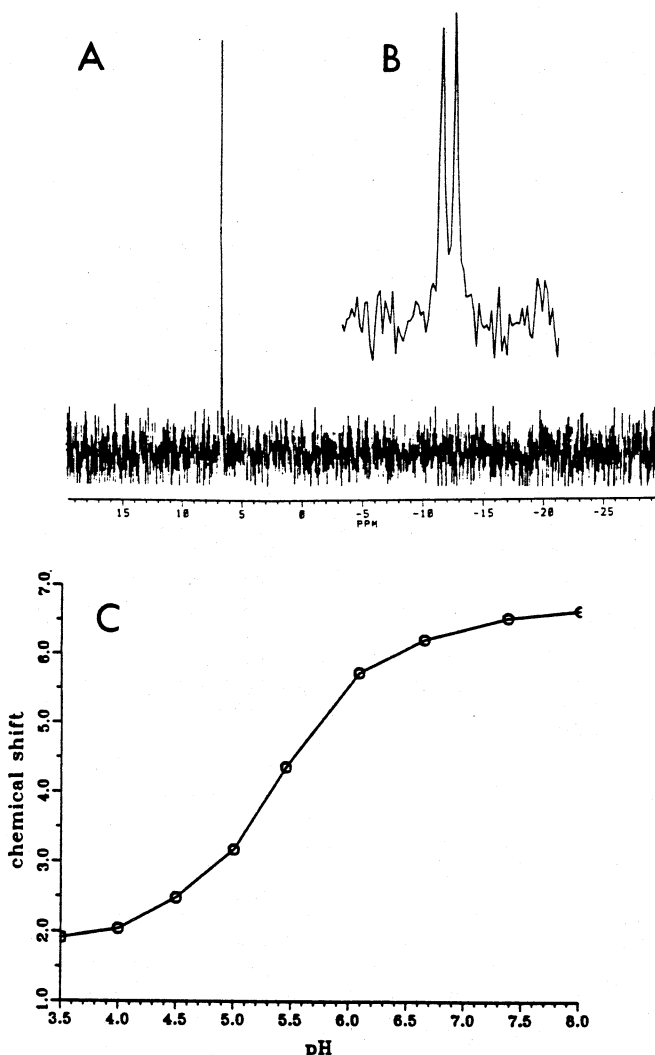


FIG. 1. A, proton-decoupled <sup>31</sup>P NMR spectrum of the material purified from calf lenses. Data were collected on a 1.5-mg sample in 1.3 ml of D<sub>2</sub>O, pH 7.5, using a 10-mm microcell in a standard 10-mm <sup>31</sup>P probe. Acquisition parameters were: pulse width, 8  $\mu$ s (60°); number of scans, 16; repetition time, 1.4 s; sweep width, 12.5 kHz. Data were transformed using a 3-Hz line broadening filter. B, <sup>1</sup>H-coupled NMR spectrum of the material. Spectral parameters are the same as in A. C, pH titration of the <sup>31</sup>P chemical shift of the compound.

which are typical of carbons with a single hydroxyl group. No anomeric (95–100 ppm) or carbonyl (195–220 ppm) carbons are present in the molecule. The <sup>1</sup>H-coupled carbon spectrum (Fig. 2B) shows that the two resonances at 63.5 and 64.5 ppm are triplets and the four downfield peaks are doublets accounting for eight nonexchangeable protons. These results suggest that the compound is a polyol phosphate.

The proton spectrum of the material is complex (Fig. 3) but extends only over 0.65 ppm from a resonance at 4.2 ppm to the most upfield resonance at 3.55 ppm, a chemical shift range consistent with the compound being a carbohydrate. By heteronuclear <sup>31</sup>P-<sup>1</sup>H two-dimensional spectroscopy and selective proton-decoupling experiment (data not shown) we have identified the multiplet at 4.2 as the <sup>31</sup>P-coupled proton. Integration of the proton resonances, normalizing to the 4.2-ppm resonance as one proton, is consistent with the count of eight nonexchangeable protons obtained from the <sup>13</sup>C experiment. In the spectrum, each of resonances A through E represents one proton; resonance F represents three overlapping protons. Among these peaks, two (C and E) are doublets of doublets

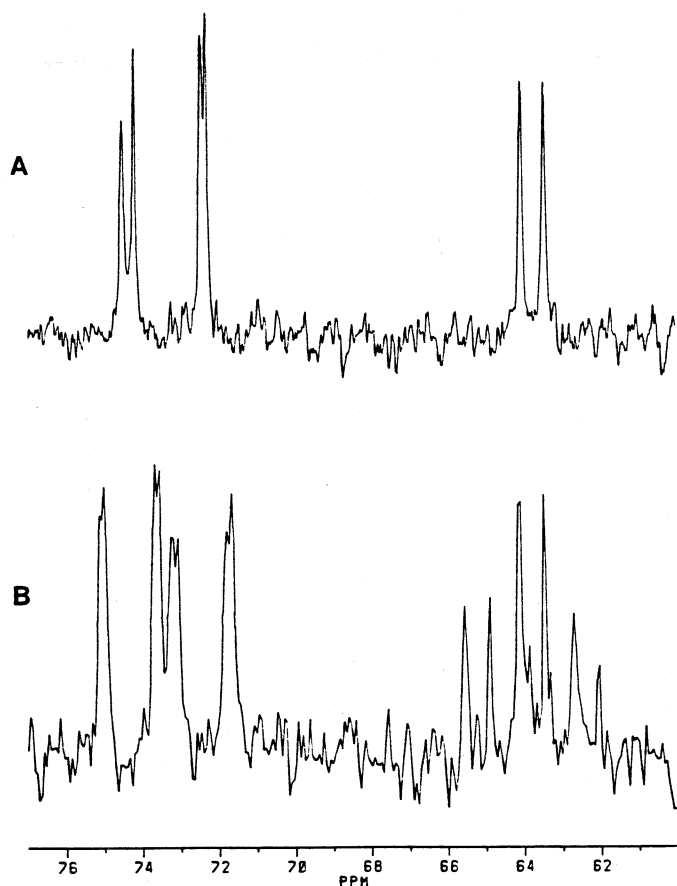


FIG. 2. A, proton-decoupled  $^{13}\text{C}$  NMR spectrum of the compound. Sweep width, 5000 Hz; pulse width,  $6\ \mu\text{s}$  ( $45^\circ$ ); relaxation delay, 1.4 s; number of scans, 70,000. Fourier transformed after application of a 5-Hz line broadening function. B,  $^1\text{H}$ -coupled  $^{13}\text{C}$  NMR spectrum of the material. 50,000 scans; other parameters are the same as above. The small shifts of the downfield peaks are due to minor temperature differences between the decoupled (A) and coupled (B) spectra.

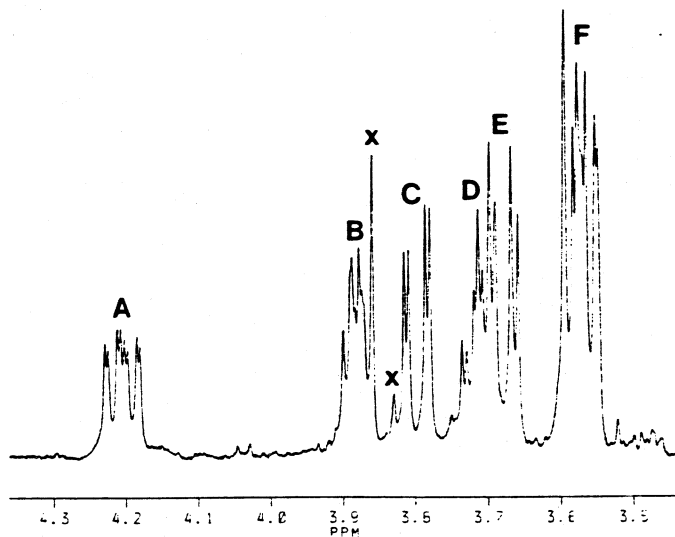


FIG. 3.  $^1\text{H}$  NMR spectrum of the unknown. Resonances marked by x are due to impurities in the sample. Data were obtained in 400 scans with  $4\text{-}\mu\text{s}$  pulses ( $45^\circ$ ), 3-s repetition delay, and a sweep width of 750 Hz.

whose larger coupling constant (11.7 and 12.0 Hz, respectively) is highly characteristic of geminal protons.

This identification of geminal protons was confirmed by a

$^1\text{H}$  homonuclear COSY spectrum acquired with a  $45^\circ$  mixing pulse. As described by Bax (21), in this type of a spectrum, based on the tilt angle of the cross-peaks, one can distinguish between geminally coupled protons with their negative  $J$  couplings and vicinally coupled protons with positive  $J$  couplings. Thus, in Fig. 4A, if one connects the centers of gravity of the quartets composing the cross-peaks at 3.67 and 3.80 ppm, one obtains lines which form angles of less than  $45^\circ$  with the diagonal (see the two upper rightmost cross-peaks in Fig. 4A). This is indicative of geminal proton cross-peaks. In contrast, similar analysis of other cross-peaks results in lines forming angles greater than  $45^\circ$  with the diagonal, indicative of vicinal coupling.

Fig. 4B is a contour plot of a conventional COSY spectrum using a  $90^\circ$  mixing pulse. Using this spectrum together with a phase-sensitive double quantum two-dimensional spectrum (22, 23) (data not shown), we have determined the entire connectivity pattern of the molecule, leading to a sequential assignment of all its protons starting with the geminal proton 1a (Table I). Based on the above analyses, we concluded that the unknown was a six-carbon polyol, phosphorylated on the third or fourth carbon.

The parent polyol was obtained by hydrolyzing the purified material with alkaline phosphatase. The polyol thus obtained had  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra identical with that of sorbitol. This NMR identification was further substantiated by demonstrating that the dephosphorylated unknown and sorbitol were identical by gas chromatography and gas chromatography/mass spectroscopy. The absolute configuration of this material was determined to be D-sorbitol by an enzymatic assay with sorbitol dehydrogenase specific for D-sorbitol.

Comparison of the  $^{13}\text{C}$  spectrum of the phosphate ester (Fig. 5A) with that of sorbitol (Fig. 5, B and C) suggested that the location of the phosphate ester is the C3 of sorbitol since this is the only carbon peak whose position shifts substantially following dephosphorylation. To confirm this assignment, we synthesized sorbitol 3-phosphate from glucose 3-phosphate (18) by reduction with sodium borohydride.  $^{31}\text{P}$  NMR was used to follow the rate of reduction and showed a smooth conversion of glucose 3-phosphate anomers (4.89 and 4.95 ppm) to sorbitol 3-phosphate (6.5 ppm) and the absence of any other phosphorus-containing compounds. Sorbitol 3-phosphate was obtained as the ammonium salt after purification by anion exchange chromatography. This material was identical to that obtained from the lens in its  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{31}\text{P}$  NMR spectra and titrated with an identical  $\text{pK}_a$ .

To verify that the compound at 6.5 ppm in diabetic lens is sorbitol 3-phosphate, an extract of such rat lenses (kindly provided to us by Drs. Gonzalez and Cheng, Harvard Medical School) was spiked with synthetic Sor-3-P.<sup>1</sup> Both coupled and decoupled  $^{31}\text{P}$  spectra of the extract after the spike (data not shown) demonstrate that only a single compound is present, confirming that sorbitol 3-phosphate is indeed the material observed in diabetic rat lenses.

Identification of the unusual lenticular metabolite as sorbitol 3-phosphate raises interesting questions about the biosynthetic pathways leading to the production of this compound and its possible function. Possible pathways for the synthesis of Sor-3-P are: (a) direct phosphorylation of sorbitol by an appropriate kinase; (b) reduction of an appropriately phosphorylated precursor such as fructose 3-phosphate or glucose 3-phosphate; (c) an aldol-type condensation from trioses. Based on the current understanding of the aldol reductase pathway in the lens, it is not possible to decide which of these alternatives is in fact operative.

<sup>1</sup> The abbreviation used is: Sor-3-P, sorbitol 3-phosphate.

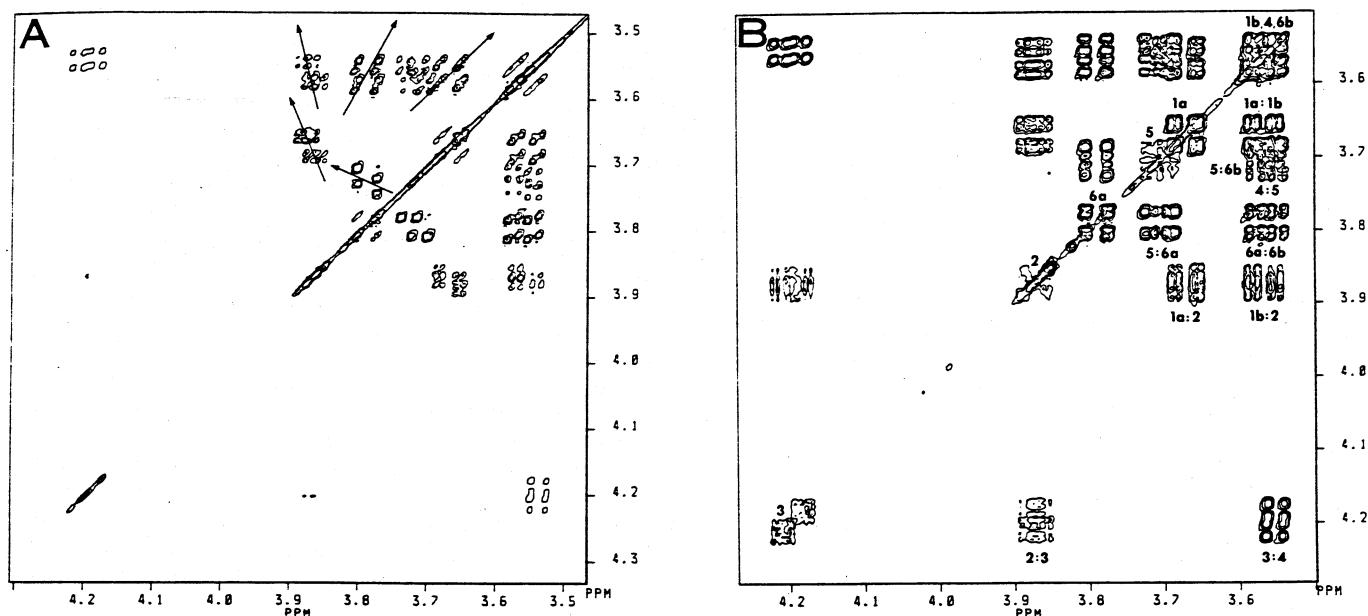


FIG. 4. A,  $^1\text{H}$  COSY spectrum obtained with  $45^\circ$  mixing pulse. Upper cross-peaks at 3.67 and 3.8 ppm (horizontal axis) are due to geminal couplings. Other cross-peaks are due to vicinal couplings. Data were acquired as a  $512 \times 1024$  matrix with 64 scans per each time point; pulse width,  $6 \mu\text{s}$  ( $60^\circ$ ); relaxation delay, 3.75 s; sweep 2, 700 Hz; sweep 1, 750 Hz. Data were transformed in both dimensions using an unshifted sine bell filter. B,  $^1\text{H}$  COSY spectrum obtained with a  $90^\circ$  mixing pulse. Protons are identified according to their location in the molecule. Proton couplings giving rise to each cross-peak are indicated next to each peak. Thus, the interaction between protons 1a and 2 is denoted as 1a:2 next to the appropriate cross-peak. Data were obtained as a  $1024 \times 1024$  matrix; other parameters are the same as in A.

TABLE I

Proton chemical shifts and coupling constants of sorbitol 3-phosphate

Proton	Chemical shift	Couplings
	ppm	Hz
1a	3.76	$J_{1a-1b} = -12.0$ , $J_{1a-2} = 3.8$
1b	3.57	$J_{1b-1a} = -12.0$ , $J_{1b-2} = 5.5$
2	3.87	$J_{2-1a} = 3.8$ , $J_{2-1b} = 5.5$ , $J_{2-3} = 6.8$
3	4.20	$J_{3-2} = 6.8$ , $J_{3-4} = 1.9$ , $J_{P-H} = 10.5$
4	3.55	$J_{4-3} = 1.9$ , $J_{4-5} = 8.0$
5	3.70	$J_{5-4} = 8.0$ , $J_{5-6a} = 2.5$ , $J_{5-6b} = 6.6$
6a	3.79	$J_{6a-5} = 2.5$ , $J_{6a-6b} = -11.7$
6b	3.57	$J_{6b-5} = 6.6$ , $J_{6b-6a} = -11.7$

Although hypothetically the simplest and most direct pathway to sorbitol 3-phosphate is the direct phosphorylation of sorbitol at the 3 position, such activity has never been reported previously, and in preliminary experiments this kinase activity was not detected in crude extracts from rabbit lens.<sup>2</sup>

The second alternative would also involve an unusual kinase as well as a reduction of the phosphorylated product to Sor-3-P. Although neither fructose-3-phosphate kinase nor glucose-3-phosphate kinase has been reported in the lens, the ability of aldose reductase to reduce glucose 6-phosphate to sorbitol 6-phosphate (4, 24) requires that this pathway be considered.

The third alternative, although the most indirect, cannot be excluded *a priori* since some aspects of the aldose reductase pathway are at present unclear. As presently understood, in this pathway glucose is initially reduced by an NADPH-dependent enzyme aldose reductase to sorbitol (6, 7) which is in turn converted to fructose by an NAD-dependent sorbitol dehydrogenase (8). Part of the resultant fructose diffuses out of the cell (25), and a substantial fraction is phosphorylated to fructose 1-phosphate by a ketohexokinase (9, 26). Fructose

1-phosphate is then cleaved by an aldolase to dihydroxyacetone phosphate and glyceraldehyde (26). Two open questions in this scheme are the fate of glyceraldehyde and the effect of the altered redox potential in the diabetic state. The elevation in the concentrations of Sor-3-P and the unidentified compound at 5.8 ppm may be one of the consequences of changes in the glyceraldehyde concentrations and redox potential in the diabetic lens.

The precise structure of the Sor-3-P molecule which leads to its unique  $^{31}\text{P}$  NMR properties is unclear. One likely explanation is that the hydroxyls of sorbitol act to cage the phosphate through a network of hydrogen bonds which would tend to strain the C-O-P bond leading to a downfield shift of the phosphorus resonance (27). It would also explain the mildly acidic  $\text{pK}_a$  of the compound and the significant differences in the  $^1\text{H}$  spectra between Sor-3-P and sorbitol.

The identity of the second compound at 5.8 ppm which has been observed only in the diabetic lens is intriguing. Previous data (1) suggest a possible precursor-product relationship between this compound and Sor-3-P. The most obvious candidate compound for such a precursor is glucose 3-phosphate. This has been ruled out, however, because the  $^{31}\text{P}$  chemical shifts of the anomers of glucose 3-phosphate are 4.89 and 4.95 ppm (at pH 7.5). Another obvious possibility for the identity of the 5.8-ppm compound is that it is a positional isomer of sorbitol phosphate because of its unusual  $^{31}\text{P}$  chemical shift,  $^1\text{H}$ - $^{31}\text{P}$  coupling constant ( $J = 10.5$  Hz), and  $\text{pK}_a$  (5.5). This possibility was eliminated by the chemical nonselective phosphorylation of sorbitol at positions 2, 3, 4, and 5 which gave four isomeric phosphates, none of which resonated at 5.8 ppm. Positive identification of this compound will, therefore, have to await its purification.

In conclusion, we have identified the previously unknown phosphomonoester observed in normal and diabetic lenses as D-sorbitol 3-phosphate. To extend this finding, we intend to

<sup>2</sup> B. Szwergold, unpublished observations.

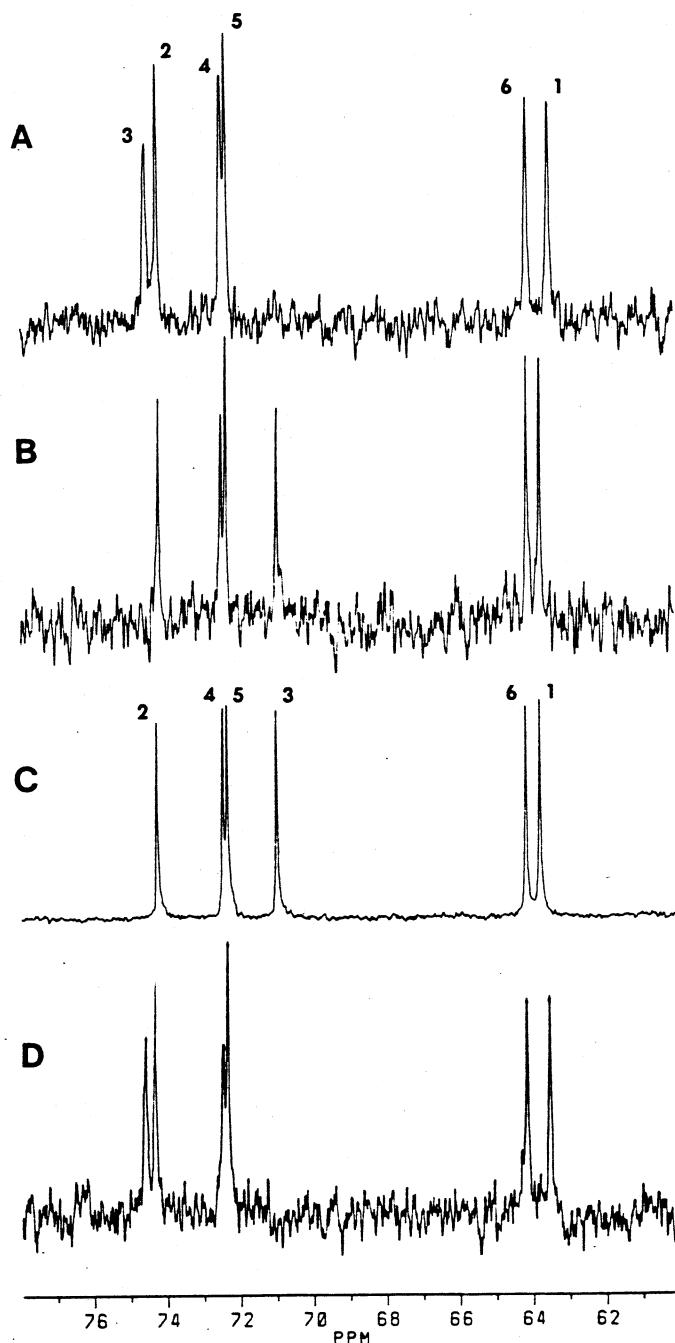


FIG. 5. A, proton-decoupled  $^{13}\text{C}$  NMR spectrum of the phosphate ester (pH 6.5). Obtained with 8- $\mu\text{s}$  pulses; 1.4-s relaxation time; sweep width, 5000 Hz; and 70,000 scans. Processed with a 5-Hz line broadening. B, proton-decoupled spectrum of the dephosphorylated material. Acquisition parameters are the same as above except for the number of scans, 40,000. C, decoupled  $^{13}\text{C}$  spectrum of sorbitol. Parameters are the same as above; 64 scans. D,  $^1\text{H}$ -decoupled  $^{13}\text{C}$  spectrum of synthetic Sor-3-P. Number of scans, 3000; other parameters are the same as in A.

address the following questions: (a) what are the biosynthetic pathways of this unusual phosphate; (b) is the compound with a  $^{31}\text{P}$  resonance at 5.8 ppm related to Sor-3-P; (c) what is the connection between these compounds and the elevated levels of  $\alpha$ -glycerophosphate in the diabetic lens; (d) can similar compounds be found in other tissues with high levels of aldose reductase; and (e) do these products have a function in the cell and do they play a role in diabetic cytopathy or are they merely dead end by-products?

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#### REFERENCES

- Gonzalez, R. G., Miglior, S., Von Saltza, I., Buckley, L., Neuringer, L. J., and Cheng, H. M. (1988) *Magn. Res. Med.* **6**, 435–444
- Gonzalez, R. G., Barnett, P., Cheng, H. M., and Chylack, L. T., Jr. (1984) *Exp. Eye Res.* **39**, 553–562
- Willis, J. A., and Schleich, T. (1986) *Exp. Eye Res.* **43**, 329–341
- Sarges, R., Schnur, R. C., Belletire, J. L., and Peterson, M. J. (1988) *J. Med. Chem.* **31**, 230–243
- van Heyningen, R. (1959) *Nature* **184**, 194–195
- Flynn, T. G. (1986) *Metabolism* **35**, (suppl.) 105–108
- Srivastava, S. K., Ansari, N. H., Hair, G. A., Awasthi, S., and Das, B. (1986) *Metabolism* **35**, (suppl.) 114–118
- Jeffrey, J., and Jornvall, H. (1988) *Adv. Enzymol.* **61**, 47–106
- Ohrloff, C., and Hockwin, O. (1973) *Ophthalmic Res.* **5**, 121–128
- Kador, P. F. (1988) *Med. Res. Rev.* **8**, 325–352
- Kopp, S. J., Glonek, T., and Grenier, J. V. (1982) *Science* **215**, 1622–1625
- Bax, A., and Morris, G. A. (1981) *J. Magn. Reson.* **42**, 501–505
- Kieboom, A. P. G., Sinnema, A., van der Toorn, J. M., and van Bakkum, H. (1977) *Recl. Trav. Chim. Pays-Bas Belg.* **96**, 35–37
- Angyal, S. J., and Le Fur, R. (1980) *Carbohydr. Res.* **84**, 201–209
- Aue, W. P., Bartholdi, E., and Ernst, R. R. (1976) *J. Chem. Phys.* **64**, 2229–2246
- Nagayama, K., Kumar, A., Wütrich, K., and Ernst, R. R. (1980) *J. Magn. Reson.* **40**, 321–334
- Bergmeyer, H. V., Gruber, W., and Gutmann, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. V., ed) pp. 1323–1326, Academic Press, New York
- Brown, D., Hayes, F., and Todd, A. (1957) *Chem. Berichte* **90**, 936–941
- Tener, G. M. (1961) *J. Am. Chem. Soc.* **83**, 159–168
- Gadian, D. G., Radda, G. K., Richards, R. E., and Seeley, P. J. (1979) in *Biological Applications of Magnetic Resonance* (Shulman, R. G., ed) pp. 531–535, Academic Press, New York
- Bax, A. (1984) in *Two-dimensional Nuclear Magnetic Resonance in Liquids*, pp. 82–84, D. Reidel Publishing Company, Dordrecht, Holland
- Marion, D., and Wütrich, K. (1983) *Biochem. Biophys. Res. Commun.* **113**, 967–974
- Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., and Wütrich, K. (1983) *Biochem. Biophys. Res. Commun.* **117**, 479–485
- Srivastava, S. K., Ansari, N. H., Brown, J. H., and Petrash, J. M. (1982) *Biochim. Biophys. Acta* **717**, 210–214
- Kuck, J. F. R., Jr., and Croswell, H. H., Jr. (1974) *Ophthalmic Res.* **6**, 189–196
- Ohrloff, C., Zierz, S., and Hockwin, O. (1982) *Ophthalmic Res.* **14**, 221–229
- Gorenstein, D. G. (1984) in *Phosphorus-31 NMR* (Gorenstein, D. G., ed) pp. 7–36, Academic Press, Orlando, FL